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Glycosphingolipids govern gene expression

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To elucidate the biological significance of the lactosylceramide (LacCer) branching in glycosphingolipid (GSL) biosynthesis, we established ganglioside GM3- and lactosylsulfatide SM3-reconstituted cells by introducing the GM3 synthase gene and the sulfotransferase gene, respectively. In SM3-expressing cells, the reduction of β1 integrin mRNA expression, the reduced adhesivity to fibronectin and laminin, and the suppression of anchorage-independent growth (tumorigenic potential) were observed. On the other hand, in GM3-expressing cells, anchorage-independent growth was promoted and the expression of PDGFα receptor mRNA was specifically reduced. Interestingly enough, no change in anchorage-dependent growth was observed in these cells, and tumorigenic signals were controlled selectively in both positive and negative directions. Thus, the spatio-temporal, gene expression control mechanism by individual GSL molecules accumulating in the cell membrane microdomain (raft) has been proven.
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Keywords: **ganglioside GM3, sulfatide SM3, malignancy, PDGFα receptor, β1 integrin**

Introduction

The microdomain, a supra-biomolecular complex on the cell membrane, is a minute region that consists of aggregates of glycosphingolipids (GSLs), sphingomyelins, and cholesterol. It also consists of various signaling molecules such as Src family kinases and transmembrane-type receptors. Because of its leading role in mediating extracellular signals, the elucidation of the mechanism of the microdomain has been attracting worldwide attention. Although GSLs are assumed to function as a regulator of various proteins in the microdomain, obvious lack of knowledge concerning its specific mechanism of action remains to date. In conventional studies of GSL function, various discussions were held based on GSL depletion experiments, *e.g.*, by exogenous addition of individual GSL molecules into cultured cells and using GSL biosynthesis inhibitors, and so forth. A definite answer, however, remains to be obtained. By using GSL-reconstituted cells to which various GSL synthase genes have been introduced and pathological cell models, we have verified the potential roles of the GSL molecules of the microdomain to actively control gene expression by regulating the transmembrane signal transduction.

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Regarding the structural variation of GSLs, the expression of five enzymes acting on lactosylceramide (LacCer) is the rate-determining step (Figure 1). Starting with the GA2/GM2/GD2 synthase [1] cloned in 1992, followed by the cerebroside sulfotransferase (CST) gene [2], the GM3 synthase (SAT-1) gene [3], the globoside synthase gene [4], the isoglioboside synthase gene [5], and finally in 2001, the aminoCTH synthase (β3Gn-T5) gene [6], which is the key enzyme of lacto/neolacto type GSLs. We have been working on the elucidation of the functions of individual GSL molecules by using various GSL-reconstituted cells and have prepared by introducing genes that act on the LacCer branching into LacCer high-expression cells that are cloned from wild type 3LL Lewis lung carcinoma cells [7]. To understand the physiological and pathological functions of GSL molecules, which would reflect their dynamic intracellular activities, such as microdomain formation and function, vesicle transport and internalization, it is extremely important to utilize GSL-reconstituted cells expressing individual GSL selectively which share with a common genetic background.

In this paper, we describe our recent studies on the functional changes of GM3- and lactosylsulfatide (SM3)-reconstituted cells, which are prepared by introducing the SAT-1 gene [3] and the cerebroside sulfotransferase (CST) gene [2], respectively. The transformed phenotypes of these GSL-reconstituted 3LL lung carcinoma cells are promoted and suppressed by endogenous GM3 and SM3 [8,9], respectively.

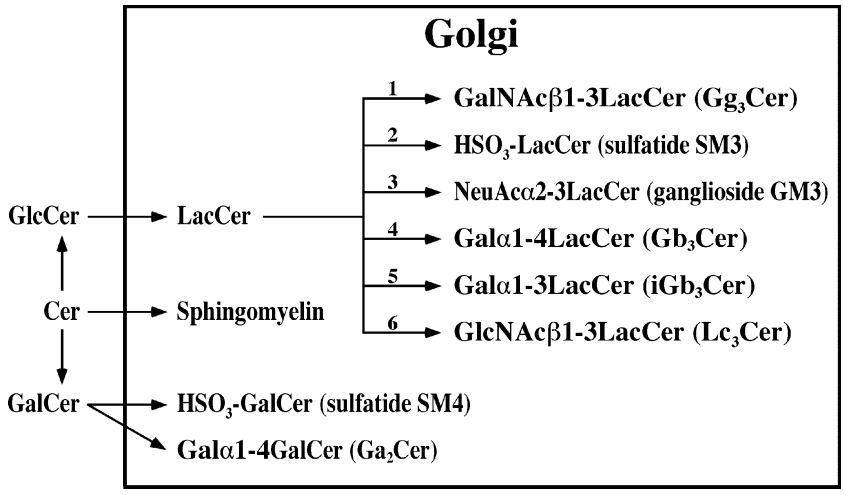

Figure 1. LacCer branching in GSL biosynthesis Enzymes acting on LacCer; 1. GA2/GM2/GD2 synthase (GalNAc-T) [1]; 2. cerebroside sulfotransferase (CST) [2]; 3. GM3 synthase (SAT-I) [3]; 4. Gb3 synthase [4]; 5. iGb3 synthase [5]; 6. Lc3 synthase (β3Gn-T5) [6].

Regulation of integrin expression and tumorigenesis by lactosylsulfatide SM3

SM3-reconstituted cells

SM3 is biosynthesized by transferring sulfate onto the galactose residue of LacCer by the action of CST [3] localized in the cavities of Golgi apparatus. In addition to LacCer, CST catalyzes the biosynthesis of galactosylceramide sulfate (SM4) and seminolipid (SM4g), using galactosylceramide and galactosyl alkylacyl glycerol as the substrates, respectively. These sulfo- glycolipids are collectively referred to as "sulfatides" a term, however, often used to indicate SM4 in a narrow sense. While SM4 has been studied in various fields as the major component of the myelin sheath, hardly any attempt has been made to elucidate the physiological function of SM3. Inokuchi *et al.* previously obtained a J5 clone from 3LL Lewis lung carcinoma cells derived from spontaneous lung carcinoma cells of C57BL6 mouse, which express LacCer at a high level but lack galactosylceramide, lacto/neolacto-series, sulfo-series, and ganglio-series [7]. Here we established GSL-reconstituted cells stably expressing SM3 by introducing the CST gene into J5 cells (J5/CST cells) (Figure 2) [9].

Endogenous SM3 selectively suppresses β1 integrin gene expression and cell adhesion

J5/CST cells showed normal cell proliferation (anchorage-dependent growth) which is exactly the same as that of mock

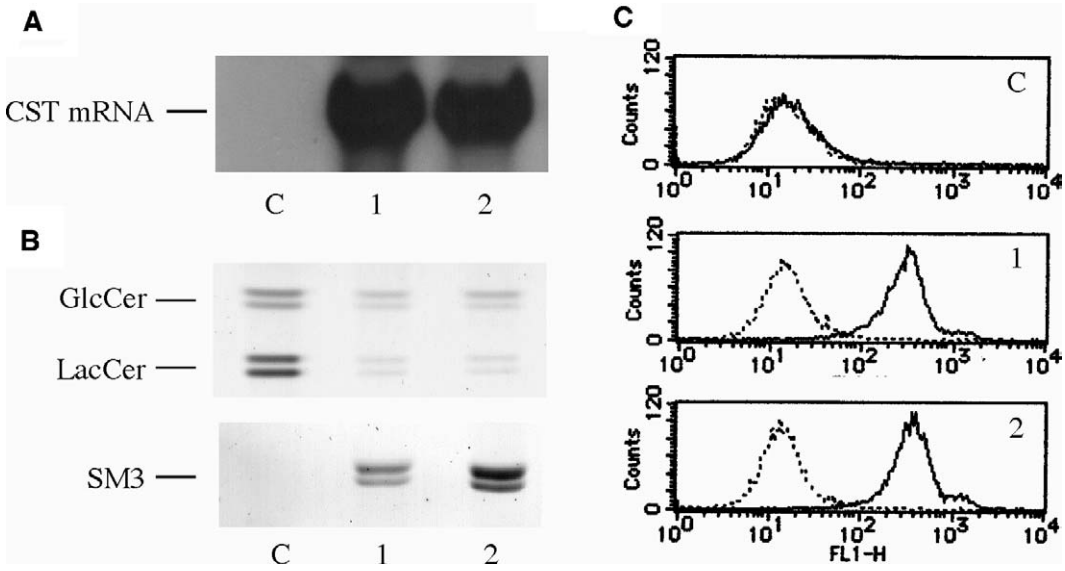

Figure 2. Establishment of SM3-reconstituted cells. (A) Northern blot analysis of CSTmRNA; (B) GSL analysis by HPTLC; (C) cell surface expression of SM3 by flow cytometry. (C) mock transfected cells; 1, J5/CST-1; 2, J5/CST-2.

Table 1. Flow cytometric analysis of the amounts of cell surface and total cellular integrins. The values were expressed as geometric mean fluorescence intensity

	Cell surface			*Total*		
	Mock	*J5/CST-1*	*J5/CST-2*	*Mock*	*J5/CST-1*	*J5/CST-2*
$\alpha 5$	130.8	87.9	77.8	243.3	97.9	147.6
$\alpha 6$	198.8	113.1	114.1	503.5	336.4	260.8
$\beta 1$	199.1	121.0	134.6	327.1	196.6	172.8
H-2D^b	24.1	34.5	33.9	111.0	98.0	88.1
H-2K^b	18.4	19.1	17.7	227.6	194.8	232.1

transfected cells (Figure 3A), except the shape is spherical and a decrease in extensibility on the plastic plate can be observed (Figure 3B). A study of the adhesivity to fibronectin and laminin revealed a significant decrease compared with mock transfected cells (Figure 3C). When the expression levels of $\alpha 5$, $\alpha 6$, and $\beta 1$ integrin subunits on cell surface were measured by flow cytometry, all of integrin subunits were found to decrease in J5/CST cells (Table 1). Decreases in the total integrin expression levels were also observed after membrane permeation treatment (Table 1). When the $\alpha 5$ and $\beta 1$ subunits were subjected to Northern blotting to determine gene expression of integrin molecules, it was interestingly revealed that only $\beta 1$ integrin mRNA expression was decreased (Figure 4A). From the comparison of $\beta 1$ integrin mRNA expression levels with three SM3-reconstituted cells exhibiting different levels of SM3, a clear negative correlation was observed in the expression level between SM3 and $\beta 1$ integrin mRNA (Figure 4B). From these results, it was assumed that in the SM3-expressing cells, the

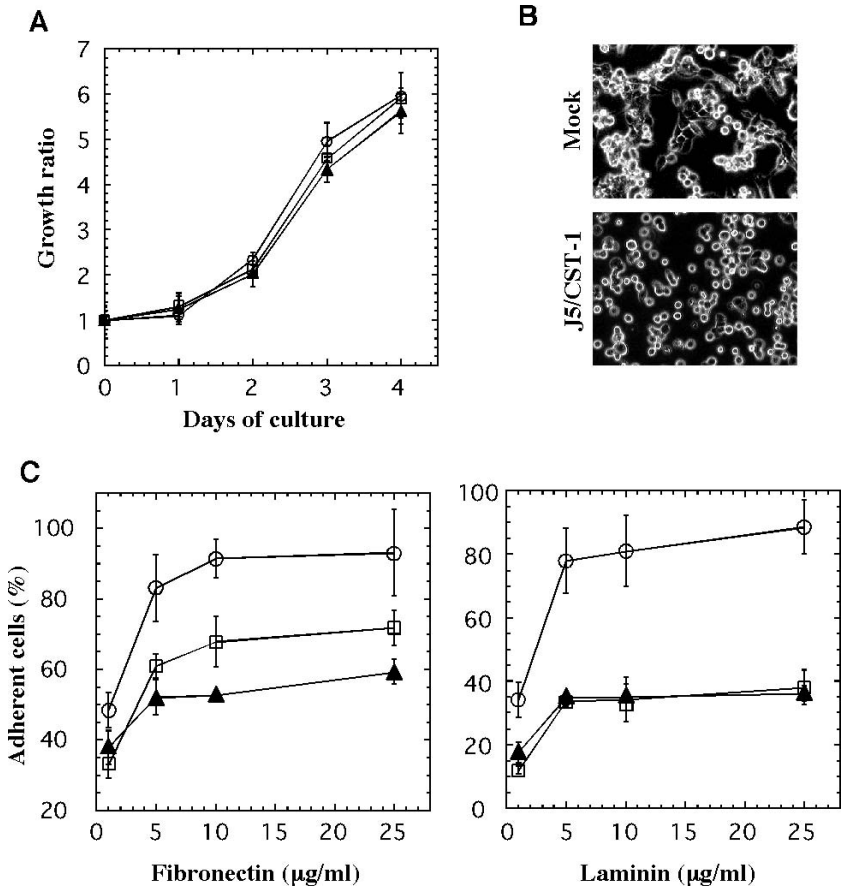

Figure 3. Growth behavior, morphlogy and cell attachment to extracellular matrix proteins of SM3-reconstituted cells. (A) proliferation under normal culture conditions (anchorage-dependent growth in the presence of 10% FCS; (B) round and spherical morphology of J5/CST compared to mock; (C) cell adhesion to fibronectin and laminin. Mock (○), J5/CST-1 (□), J5/CST-2 (▲).

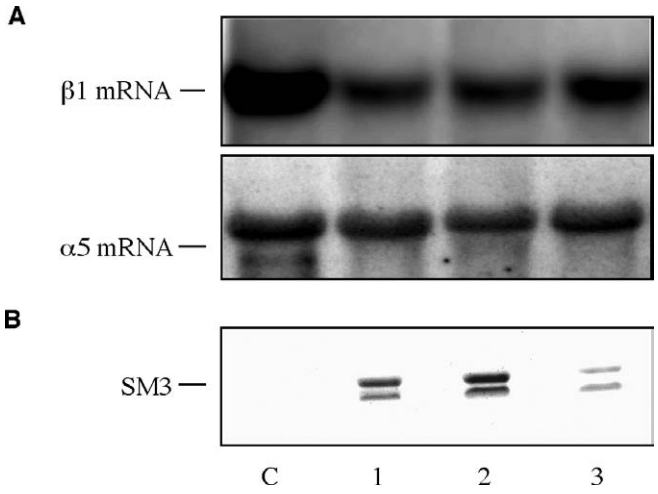

Figure 4. Inverse relationship between the level of SM3 and β1 integrin mRNA. (A) Northern blot analyses of β1 integrin; (B) that of α5 integrin. Methylene blue staining of 18S and 28S rRNA in the same menbrane; (C) Comparison of SM3 contents among the CST-transfectants by HPTLC.

biosynthesis level of β1 integrin is selectively suppressed, and therefore the efficient formation of the integrin $\alpha\beta$ heterodimer in the endoplasmic reticulum is impeded. Because of this, excess α subunits are transferred into the decomposition pathway, finally causing a decrease in the number of integrin molecules (Figure 5).

The involvement of GSL in the adhesion phenomenon of cells to an extracellular matrix has long been widely known. The phenomena that adhesion of keratinocytes to fibronectin is impeded by ganglioside GT1b [10] and that adhesion to fibronectin is promoted by the transport of extracellular GM3 into FUA169 cells [11] have been observed. In osteosarcoma cells, with GD1a expressed via sugar chain remodeling by the introduction of glycosyltransferase genes, adhesivity to vitronectin is decreased [12]. A recent study has revealed the existence of integrin molecules also in the microdomain [13], and it is surmised that their functional expression results from their association with tetraspanin membrane protein (TMP) [14]. We have found the existence of β1 integrin gene expression control mechanism by endogenous SM3 [9]. This discovery has led to a new task of clarifying the functional correlation between GSL and the cell adhesion to extracellular matrix; and further intensive studies are highly expected.

Endogenous SM3 abrogates transformed phenotypes

Although no abnormality in anchorage-dependent growth was observed in SM3-reconstituted cells J5/CST cells (Figure 3A), we observed an unexpected disappearance of the colony-forming ability (anchorage-independent growth) in soft-agar medium (Figure 6A). This anchorage-independent growth is one of the most fundamental characteristics of tumor cells, and its close relationship with tumorigenic potential

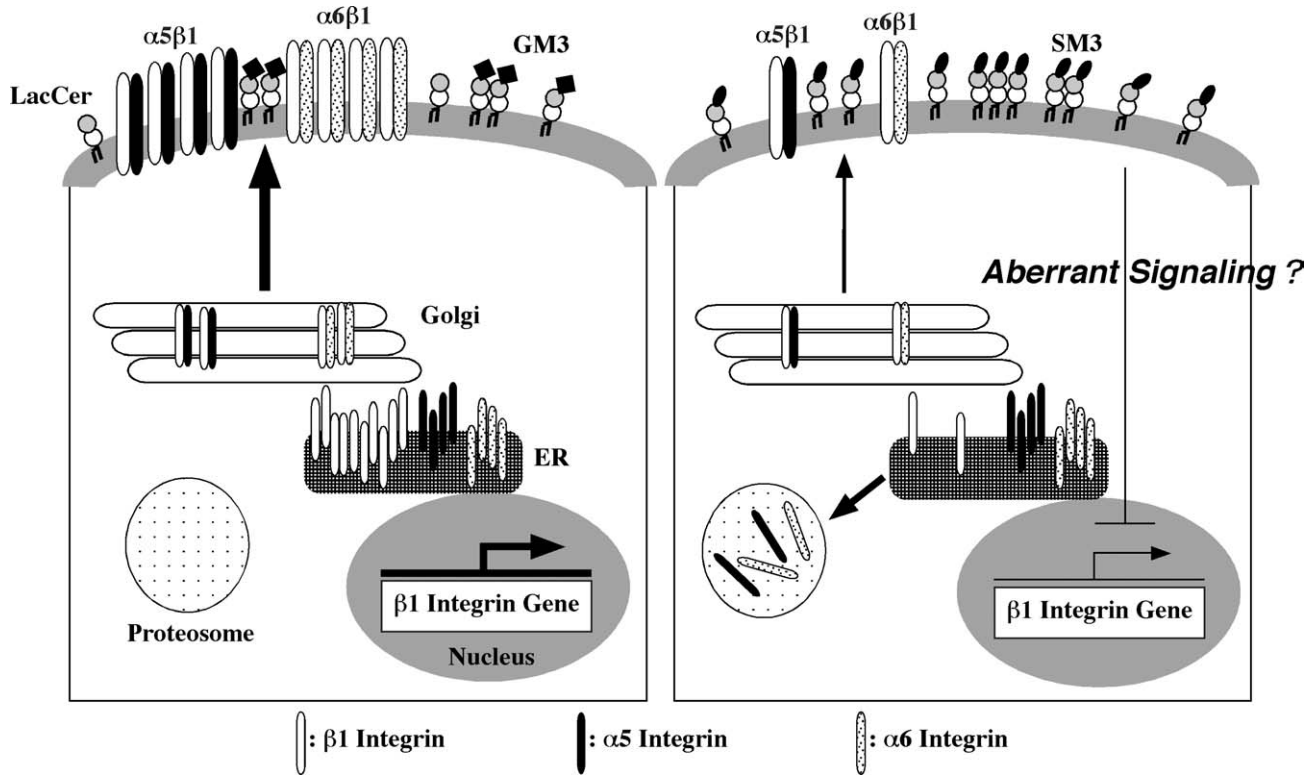

Figure 5. Working hypothesis on the regulation of integrin expression by SM3.

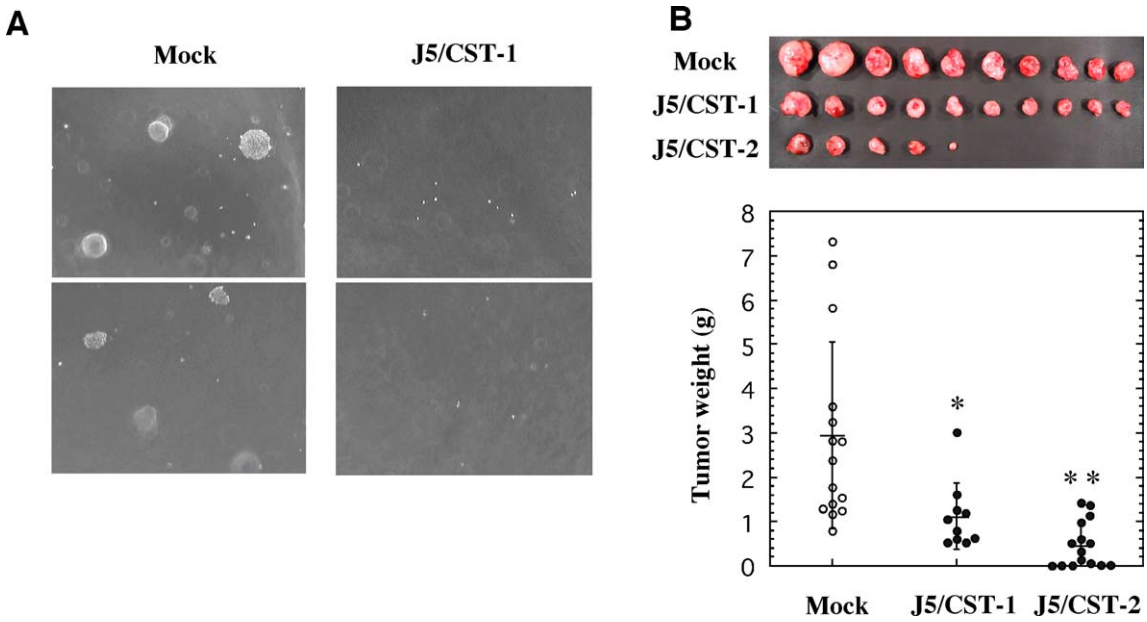

Figure 6. Loss of tumorigenic potential of SM3-reconstituted lung cancer cells *in vitro* and *in vivo* (A) Anchorage-independent growth in soft-agar medium; (B) Tumors were induced in C57/BL6 mice (8 weeks old, female) by subcutaneous armpit injection and three weeks later tumors were excised and weighed.

has been widely accepted. The SM3-expressing cells and the mock transfected cells were transplanted into the armpits of C57BL6 mice, and three weeks later tumor weights were measured. The results show either a sharp decrease in the primary tumor weight of the J5/CST cells or a complete disappearance (Figure 6B). In the SM3-reconstituted cells, no change was observed in the expression of the major histocompatibility antigen (MHC class I antigen) (Table 1) and a marked decrease of the tumorigenic potential in nude mice was also noted. All of these results suggest that the tumorigenic signals are suppressed in SM3-reconstituted cells through the lowered expression of the integrin molecules, which result in the inability to promote three-dimensional proliferation [9].

Enhancement of transformed phenotypes and suppresion of PDGFα receptor gene expression by endogenous GM3

Regulation of receptor function by gangliosides

It is well known that gangliosides are involved in the regulation of cell growth/differentiation and that the formation of malignant cells leads to changes in ganglioside composition. In general, as cells become malignant, the expression of complex gangliosides such as GM1 and GD1a is shifted into more simple gangliosides such as GM3 and GD3 [15–17]. There have been many reports concerning the possible role of gangliosides existing in cell membranes in regulating cell growth. For example, GM3 suppresses cell growth induced by epidermal growth factor (EGF) in A431 human epidermoid carcinoma

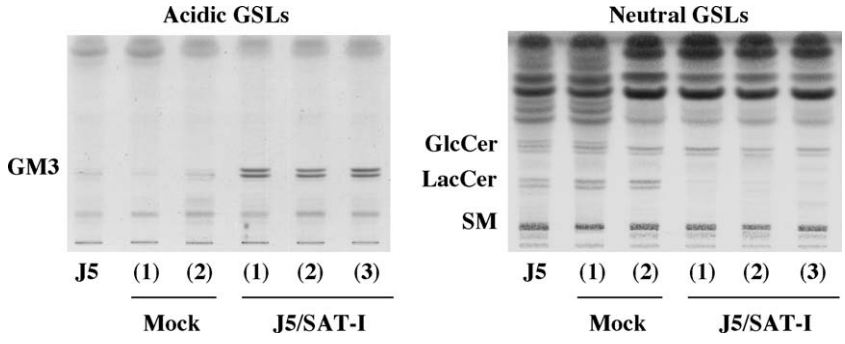

Figure 7. Establishment of GM3-reconstituted cells.

cells as well as that induced by basic fibroblast growth factor (bFGF) in baby hamster kidney (BHK) cells [18,19]. GM1 in 3T3 fibroblasts suppresses cell growth induced by a platelet-derived growth factor (PDGF) [20,21]. On the other hand, GM1 enhances the effect of nerve growth factor (NGF) in cells acting on a NGF such as PC12 cells, etc. [22]. Thus, it is assumed that gangliosides regulate receptor function and cellular interaction.

Most GSLs form microdomains by associating with cholesterol on cell membranes. It has been reported that gangliosides may regulate the functions of various signal transducing molecules that accumulate in microdomains including cytokine receptors. For example, Lyn of non-receptor-type tyrosine kinase is immunoprecipitated by anti-Gal-GD1b antibody in rat basophilic leukemia cells [23], and by anti-GD3 antibody in the rat encephalon [24]. It has also been reported that in B16 melanoma cells, c-Src, Ras, and Rho, among others, accumulate in microdomains, and are coprecipitated by the GM3 antibody which is the major ganglioside of this type of cell. Furthermore, it has been reported that these signaling molecules are activated by GSL-GSL interaction [25,26]. By using D-PDMP, which is an inhibitor of glucosylceramide synthase [27–29], we have confirmed the elimination of Src family kinase from the microdomains as GSL is depleted from 3LL Lewis lung carcinoma cells [30]. In addition, Yes, which is an Src family kinase in the microdomain, is activated by Shiga toxin that

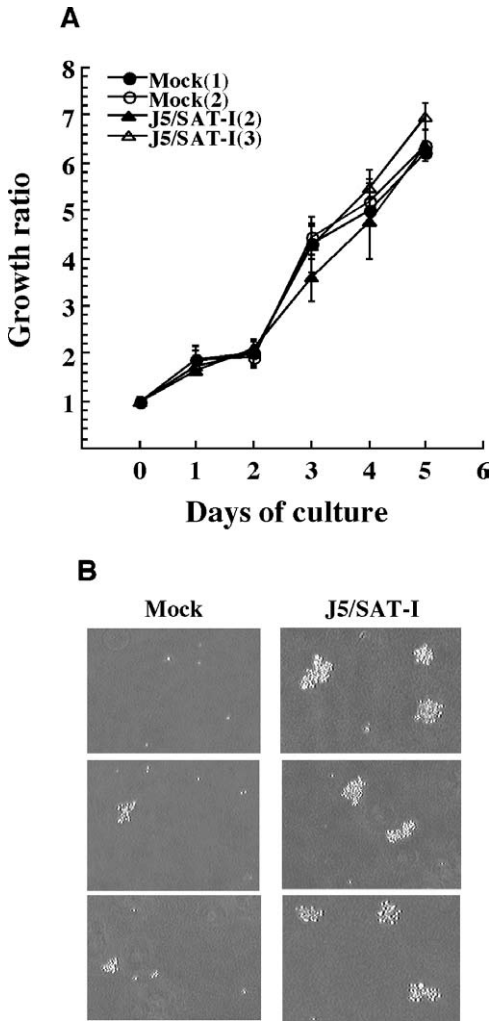

Figure 8. Enhancement of anchorage-independent growth in GM3-reconstituted cells. (A) proliferation under normal culture conditions (anchorage-dependent growth in the presence of 10% FCS; (B) Anchorage-independent growth in soft-agar medium.

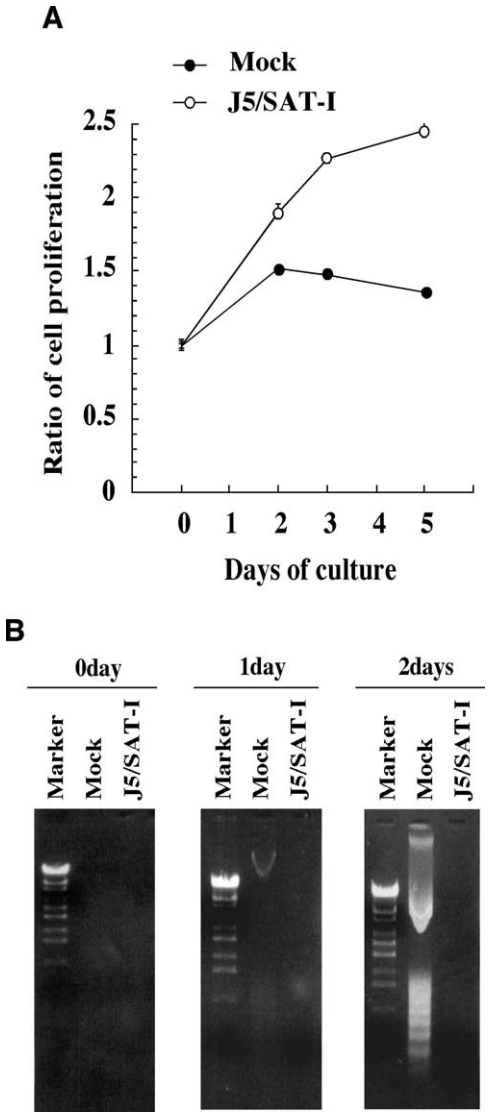

Figure 9. Cell growth and apoptosis in serum starved condition. (A) Cell growth of J5/SAT-I and in RPMI 1640 medium containing 0.1% FCS; (B) Cells were cultured in RPMI 1640 medium containing 0.1% FCS for 0, 1 or 2 days and subjected to a DNA fragmentation assay.

specifically binds to globoside Gb3 [31]. These reports strongly suggest the significance of GSL as a functional molecule in the microdomain.

Endogenous GM3 enhancement of the transformed phenotype

We established GM3-reconstituted cells (J5/SAT-1 cells) by introducing SAT-I gene into the GM3-free J5 cells [8] (Figure 7). Although, under normal culture conditions (10% serum), anchorage-dependent growth of both J5/SAT-I and mock cells was similar (Figure 8A), anchorage-independent growth of J5/SAT-I cells was significantly higher than that of mock transfected cells (Figure 8B). J5/SAT-I cells were able to proliferate progressively in the medium containing 0.1% serum compared to mock cells (Figure 9A). Moreover, under serum-starved conditions, J5/SAT-I cells displayed resistance to apoptosis whereas mock cells remained susceptible (Figure 9B). These results suggest that endogenous GM3 is needed for anchorage-independent growth, serum-independence, and anti-apoptotic properties, all of which are essential for transformed phenotypes.

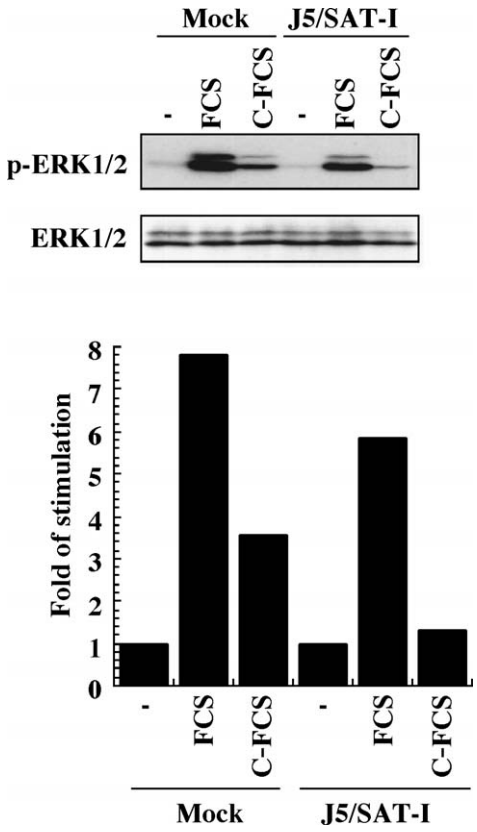

Figure 10. MAP kinase activation in GM3-reconstituted cells by FCS and C-FCS. Cells were cultured in RPMI1640 containing 0.3% FCS for 24 h. The serum-starved cells were treated with 10% FCS or 10% CFBS for 5 min, lysed and subjected to immunoblot analysis with anti-phospho-ERK1/2 antibody or anti-ERK1/2 antibody.

Suppresion of PDGFα receptor expression in GM3-reconstituted cells

ERK1/2 activity correlates well with cell proliferation acting downstream of many growth factor receptors. For this reason, we compared the ERK1/2 activation in GSL-reconstituted clones. J5/SAT-I and mock cells maintained in FCS-free medium for 24 h were subsequently stimulated with 10% FCS or lipid-depleted FCS treated with charcoal (C-FCS). ERK1/2 phosphorylation induced by FCS and CFCS in J5/SAT-I cells were 6- and 1.3-fold, respectively, but 8- and 3.5-fold in mock cells (Figure 10). The activation of ERK1/2 by lipid-depleted FCS in the GM3-reconstituted cells was remarkably low in comparison to mock cells, perhaps due to insensitivity of the cells to some polypeptide growth factors. Therefore, we examined the activation of ERK1/2 after stimulation with the polypeptide growth factors, EGF, insulin, and PDGF. Subsequently, the activation of ERK1/2 in cells stimulated with the lipid growth factors Sph-1-P and LPA. ERK1/2 activation in J5/SAT-I cells was selectively and substantially diminished by PDGF but not the other growth factors (Figure 11).

Since 3LL Lewis lung carcinoma cells express PDGFα receptor [32], its expression in J5/SAT-I cells was compared with that of mock cells. Surprisingly, there was a significant decrease in PDGFα receptor expression as measured by Western blotting (Figure 12A). To elucidate whether the decrease of PDGFα receptor in J5/SAT-I cells occurred at transcriptional or post-transcriptional level, Northern analysis was performed. Very low levels of PDGFα receptor mRNA were detected in J5/SAT-1 cells as compared to the parental J5 or mock transfected clones (Figure 12B). PDGFβ receptor mRNA was undetectable in J5, mock, and J5/CST cells by this technique (data not shown).

From these findings that anchorage-dependent growth of J5/SAT-I cells is the same as that of mock transfected cells in the presence of serum regardless of the decrease in PDGFα receptor, it can be assumed that cell growth signaling other than PDGFα receptor has complementarily been enhanced.

Concluding remarks and future prospects

Given the present circumstances in which the cloning of GSL synthase genes has nearly been accomplished, studies utilizing these genes are being conducted worldwide, based on which the elucidation of the fundamental biological significance of GSL is highly expected. In this study, we have reported for the first time that by introducing the CST gene into lung carcinoma cells and expressing SM3, β1 integrin expression is reduced at the genetic level, thereby selectively suppressing anchorage-independent growth (tumorigenic potential) without causing any change in anchorage-dependent growth [9]. At present, together with efforts toward the elucidation of its mechanism of action, we have initiated approaches to explore the potential of using CST genes in cancer therapy. At the same time, a contrasting result has been obtained in GM3-expressing cells; that is, promotion of cell

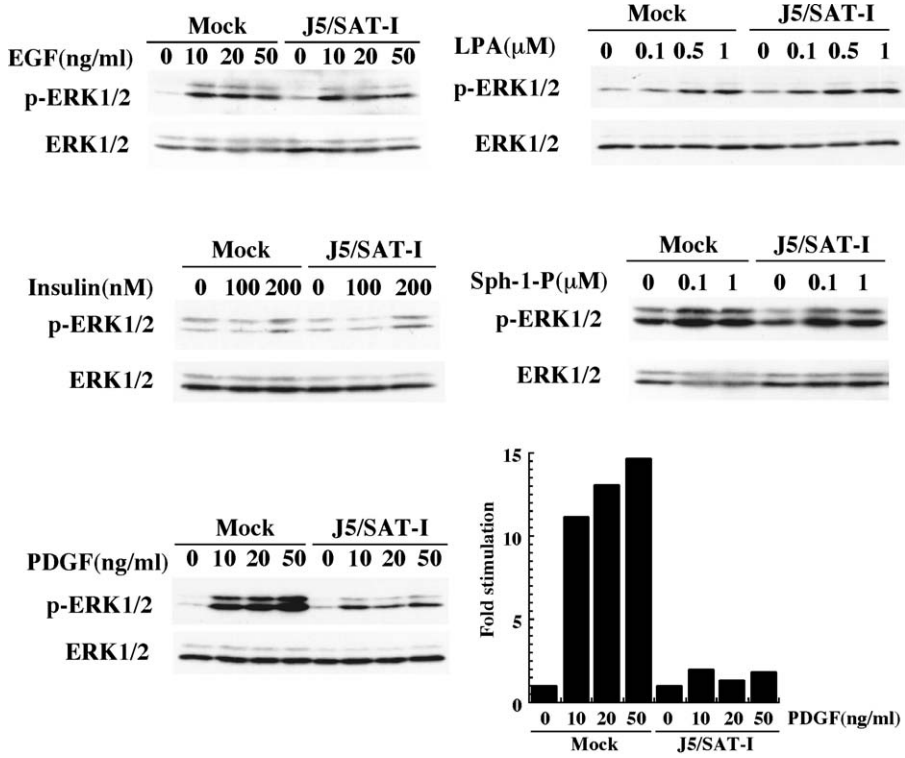

Figure 11. MAP kinase activation in GM3-reconstituted cells by EGF, insulin, PDGF, Sph-1-P and LPA. Cells were cultured in RPMI 1640 containing 0.3% FCS for 24 h. The serum-starved cells were then treated with the indicated amounts of EGF, insulin, PDGF, Sph-1-P and LPA for 5 min and lysed on lysis buffer. Lysates were subjected to immunoblot analysis with anti-phospho-ERK1/2 antibody or anti-ERK1/2 antibody.

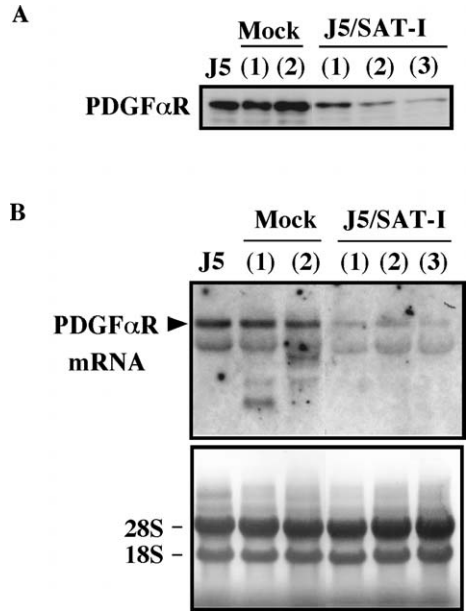

Figure 12. GM3 but not SM3 down-regulates the expression of PDGFα R. (A) Cell lysates from J5, mock and J5/SAT-I cells (top) and from J5, mock and J5/CST cells (bottom) were subjected to Western blot analysis with anti-PDGFαR. (B) Northern blot analysis of PDGFαR mRNA (top). Methylene blue staining of 18S and 28S rRNA in the same membrane (bottom).

growth and tumorigenic potential, where a decrease in the expression of PDGFα receptor mRNA has been observed [8]. As the presence of a specific gene expression control mechanism by sulfation and sialylation of the lactosylceramide branching in GSL biosynthesis [33] has been confirmed, the elucidation of its biological and pathophysiological significance will be an important subject of future investigation. What types of signal transducing mechanisms will GM3 and SM3 have accumulated in the microdomain control to bring about changes in the gene expression of integrin and the PDGFα receptor? Authors hope to contribute to life sciences in the 21st century by focusing on, as an important subject of study in the post-genome era, the elucidation of the spatio-temporal gene expression control mechanisms by glycolipid molecules.

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